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The purpose of the project is to develop a new class of antisense drug and a procedure for selecting an optimal antisense compound with HER-2/neu mRNA as the target. The scope for this year includes preparation of HER-2/neu target RNA (Task 1), development of a novel peptide synthesis technology for generation of combinatorial libraries (Task 2) and preliminary experiments on identifying members of a library that bind HER-2/neu RNA (Task 3). Task 1 has been somewhat modified in that one representative RNA fragment has been prepared, but this fragment has been further analyzed and found to have sensitive RNase H cleavage sites, which is a highly favorable result. Task 2 has been successfully completed in that a protocol for peptide synthesis in solution phase on disulfide-linked polyethylene glycol has been developed. Task 3 (months 9-12) has not yet been initiated due to unanticipated personal problems.

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Stacy Ater 7/29/98

PI - Signature Date

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Introduction

Overexpression of the HER-2/neu gene product, which is a receptor related to the epidermal growth factor receptor, is found in about one-third of breast cancers and is an independent predictor of a poor clinical outcome. Down-regulation of HER-2/neu gene expression has been proposed as a therapeutic approach for either halting proliferation of these cells or at least making them more sensitive to chemotherapy or radiation therapy. One means to accomplish this down-regulation is the use of antisense DNA compounds that can inhibit or inactivate HER-2/neu mRNA. Although antisense DNA technology has been intensively investigated in both academic and industrial laboratories over the past decade, further improvements in this technology may be required to achieve a clinically succesful product.

We have postulated a novel antisense DNA approach that may contribute to the eventual success of this technology. Our approach solves the problem of specificity, in which any particular antisense DNA drug could not discriminate sufficiently between the designated target (in this case HER-2/neu mRNA) and the myriad of similar or identical RNA sequences in a cell. Our approach is based on a dual-specificity recognition mechanism in which the antisense drug is composed of 2 parts, each of which must interact with 2 juxtaposed regions on the target mRNA (1). One part is a peptide (or peptide mimetic) that binds to a folded domain of the target mRNA, whereas the other part is a short DNA (5-mer) that is complementary to a nearby single-stranded complementary sequence. Since the DNA portion is too small to bind any complementary target by itself, it can only have antisense activity (as defined by RNase H degradation of the target) as a result of the binding avidity of the appended peptide. We have previously shown the potential utility of this novel approach using HIV-1 RNA as the target (1), but much remains to be accomplished before the value of this new therapeutic approach can be proven.

The scope for the first year of this project is to develop a process for identifying antisense DNA/peptide compounds that can be selectively targeted against HER-2/neu mRNA. In our process, combinatorial peptide libraries are synthesized in solution phase on polyethylene glycol (PEG) polymers. The PEG support has several advantages. First, it allows a more efficient synthesis of the combinatorial library, although this is not an essential feature of our approach. Second, the PEG portion of the peptide-PEG conjugate causes a large electrophoretic gel shift of target RNA, whereas a peptide by itself would cause a negligible gel shift (2). Thus, binding of conjugate to HER-2/neu mRNA can be readily observed and optimized, and gel shifted RNA can be excised from the gel to recover the bound conjugate. Third, the PEG chain is representative of and can then be replaced by an antisense DNA. That is, the steric factors that allow a PEG-linked peptide to bind its RNA target should be the same when the PEG is replaced by a short DNA strand.

In the first year of this project, we have accomplished the first 2 tasks, namely to develop the protocol for synthesizing PEG-peptide combinatorial libraries and for preparing target HER-2/neu mRNA target fragments. We have also made the important observation that the target RNA has a limited number of weak RNase H sensitive sites for short antisense DNA strands (without peptide). These sites can be further exploited when we begin working with the peptide-DNA conjugates. Unfortunately, we have not yet begun the third task planned for the first year, namely preliminary gel shift experiments with combinatorial PEG-peptide conjugates, due to the tragic death of the husband of our primary laboratory scientist, Dr. Jihong Wang.

Body

Experiment Procedure

Development of liquid-phase peptide synthesis methodology

PEG5000 was uses as a soluble support for peptide synthesis. The connection between thiol-derivatized PEG and the growing peptide was made by cysteamine through a disulfide bond. Thereby, all peptides will have a carboxy-terminal thiol group, which can be conveiently used for attachment of short oligonucleotides in a later phase of this project.

Attachment of Cysteamine to PEG-OPSS (Scheme 1).

100mg of PEG-orthopyridyl-disulfide (PEG-OPSS, FW5000, Shearwater Polymers, Inc.) was dissolved in 1ml 50mM triethylammonium acetate (TEAA) buffer (pH 8.3). 113mg of cysteamine was dissolved in 500µl H_2O to make 2M stock solution. 30ul of 2M cysteamine solution (3 times excess) was added into PEG-OPSS solution. After 5 min, 5 µl of reaction mixture was diluted to 1ml to measure UV absorption at 343 nm. More cysteamine was added to make sure the UV absorption reached the plateau, indicative of complete reaction of PEG-OPSS.

The reaction mixture was extracted with 1 ml of diethyl ether for four times. The ether layer showed a yellow color which came from the thiopyridone byproduct. Then the PEG-cysteamine in the aqueous layer was purified by size exclusion (SEC) HPLC on a BIOSEP SEC-S3000 column (300 x 7.8mm, Phenomenex, Torrance, CA). Mobile phase was 50mM TEAA (pH5). The flow rate was 1 ml/min. The first peak, corresponding to PEG-cysteamine, was collected and dried under vacuum.

Assembly of peptide (Scheme 2).

The PEG-cysteamine powder was dissolved in 500 µl of dimethyl formamide (DMF). Five times excess of activated Fmoc-amino acid derivative and HOBt were

added for each coupling reaction. The reaction was carried out at vigorous mixing for 2 hr. 0.1% bromophenol blue was added as an indicator. The color of the solution turned to yellow from blue after the free amino group reacted with the incoming protected amino acid. The reaction mixture was purified on a Sephadex LH-20 SEC column (Pharmacia, Piscataway, NJ). The void volume fraction, corresponding to the PEG-Fmoc-protected peptide, was precipitated into 10 volumes of cold ether. The precipitate was washed by cold ether for four times and dried. The Fmoc protecting group was removed by dissolving this powder into 30% piperidine/DMF and keeping for 30 min. The solution was ether precipitated and dried. The PEG-peptide was then ready for the next amino acid coupling cycle.

The split-and-pool method was adopted for preparing combinatorial libraries (Scheme 2). Thus, the PEG-cysteamine solution would be split into several portions (about 10) and each portion would be separately reacted with a different Fmoc amino acid until the bromophenol blue indicator turned yellow. The 10 separate reaction mixtures would be recombined for the SEC purification, Fmoc-deprotection and ether precipitation steps.

Removal of side-chain protecting group

After assembly, the combined peptides were deprotected with 2 ml of TFA/anisole (95/5) solution for 3 hours at room temperature. PEG-peptide conjugates was precipitated by adding dropwise into 10 times volume of cold ether (Fisher Scientific, Pittsburgh, PA). The white precipitate was washed 4 times with ether and dried. The PEG-peptides were purified by SEC on a BIOSEP SEC-S3000 column (Fig. 1). The peptides on PEG were ready for gel-shift assay.

Preparation of Target mRNA

Plasmid

Plasmid pCHC6-cerbB2 containing HER-2/neu gene was a gift from Dr. Francis Kern. It contains a 4.5kb c-erbB2 wild type human cDNA (accession no. x03363, see Appendix) as an Xba I insert in the 7.1kb expression vector pCHC6 (3). The plasmid was transformed in DH5α™ Competent E. Coli cells (GibcoBRL Life Technologies). The small scale preparation of plasmid DNA was obtained by alkali lysis and confirmed by restriction enzyme digestion (Fig. 2). The large-scale preparations of plasmid DNA were purified by Qiagen Maxi tip-500 and checked with restriction enzyme digestions (Fig. 2).

PCR

PCR was carried out in 50 µl solution in 0.5 ml tubes containing 20 mM Tris-HCl (pH8.4), 50mM KCl, 1.5 - 5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer (see Appendix), 5 ng template and 1.25 unit Taq polymerase (Promega). 30 µl of light mineral oil was layered on top of the solution. The thermal cycles started from 5 min preincubation at 94°C, followed by 25 cycles of denaturing at 94°C for 30 sec, annealling at 55°C for 30 sec, and extension at 72°C for 1 min. Final extension was at 72°C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis (Fig. 3).

In Vitro transcription

The PCR products were used to generate the mRNA *in vitro* in the presence of 40 mM Tris-HCl, pH7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 60 units of RNasin ribonuclease inhibitor, 0.5 mM of (A, U, G, C)TP, and 30 units of T7 RNA polymerase (USB). The transcription reaction was carried out at 37 °C for 3 hours. The reaction mixture (100 µl) was extracted with 100 µl of phenol:$CHCl_3$:IAA (50:49:1), The phenol layer was washed with 50 µl of DEPC-treated water, and the aqueous phases were combined and extracted with 100 µl of

$CHCl_3$. Then 15 µl of 3 M sodium acetate (pH 6) and 495 µl of ethanol were added into the aqueous phase and the RNAs were precipitated at -80 °C for 2 hours.

Gel purification of RNA

A denaturing polyacrylamide gel (5%) was prepared by dissolving 20 g of urea in 15ml H_2O, 5 ml solution of 40% acrylamide:bisacrylamide (19:1), and 4 ml 10X TBE buffer, filtering and degassing for 15 min, and pouring immediately after adding 400 µl of 10% ammonium persulfate (APS) and 20 µl N,N,N',N'-tetramethylethylenediamine (TEMED). The 5% polyacrylamide / 8 M urea gel was polymerized for 2 hours and prerun at 100 volts for 30 minutes in 1X TBE buffer to remove APS before samples were loaded.

The precipitated RNA was washed twice with 500 µl of cold 70% ethanol then dried under speed vacuum. The RNA then was dissolved in 15 µl of DEPC-treated water and 15 µl of 8M urea was added. The RNA was heated at 65 °C for 2 min, then loaded onto the gel. The gel was run at 180 V for 2 hours. The RNA band was visualized by UV light (254 nm) and cut out (Fig. 4). The RNA was electroeluted into high salt solution (3M sodium acetate, pH 6 with 0.2% BPB) from the gel slice at 90 V for 40 min. The high salt solution (400 µl) was taken out and RNA was precipitated by adding 800 µl of ethanol at -80 °C overnight.

Dephosphorylation of RNA

The precipitated RNA was washed twice with 500 µl of 70% cold ethanol then dried under speed vacuum. The RNA then was dissolved in 45 µl of DEPC-treated water and 5 µl of 10X dephosphorylation buffer was added. The reaction mixture was preheated at 50 °C for 5 min and incubated at 50 °C for 60 min after addition of 1 µl alkaline phosphatase (AP, 1 unit/µl), The RNA was phenol extracted and ethanol precipitated as described above.

Kinase labeling

The dephosphorylated RNA was washed, dried and then dissolved in DEPC-treated water. The RNA (10 pmol) was labeled by adding 12 µl of DEPC-treated water, 1.5 µl of 10X kinase buffer, 2 µl of 10 µCi [γ-^{32}P]ATP (3000 Ci/mmol, Dupont) (1 µCi ~ 2.2 X 10^6 dpm) and 1 µl of T4 polynucleotide kinase (10 unit/µl). The reaction mixture was incubated at 37 ^{o}C for 30 min then chilled on ice.

The RNAs were purified by electrophoresis on a 5% polyacrylamide / 8 M urea gel. The reaction mixtures were mixed with equal volumes of 8 M urea buffer, preheated at 65 ^{o}C for 2 min and loaded onto the gel. The gel was prerun at 100 V for 1 hour and run at 180V for 2 hours. Also, 20 µl of 8M urea with 0.025% xylene cyanol (XC) and bromophenol blue (BPB), 0.5 mM EDTA loading buffer was loaded into 3 to 4 lanes as markers.

The labeled RNA bands were located by exposing on film at room temperature for 2 to 3 min with three radioactive marks (Fig. 5). After cutting the band, the gel was re-exposed to confirm whether the right place was cut. The RNA was electroeluted to high salt solution (3M sodium acetate, pH 6 with 0.2% BPB) from the gel slice at 90 V for 40 min. The RNA was precipitated by adding two times of absolute ethanol and keeping at -20 ^{o}C for overnight. The precipitated RNA was washed twice with 500 µl of 70% cold ethanol then dried under speed vacuum. The RNA then was dissolved in 200 µl of DEPC-treated water. An aliquot (2 µl) was used for quantitating with a scintillation counter. The labeled RNAs were stored at -20 ^{o}C and could be used within two weeks.

RNase H assay

The RNase H assay was performed in buffer containing 20 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, and 5'-^{32}P-labeled

RNA (15 nM). Four oligonucleotide libraries (2-3 μM each) was added to the reaction buffer, and the mixture was incubated at 37 °C for 10 min. Digestion was started by addition of RNase H (50 units/ml, USB), and incubated at 37 °C for 2 h. Cleavage of the RNA was monitored on a 10%/8M urea-denaturing polyacrylamide gel (Fig. 6). T1 and PhyM ladders were produced by digesting RNA (~5000 cpm) with 1 μl of 1 unit/μl Nuclease T1 and Nuclease PhyM at 50 °C for 10 min after preheating in T1 sequence buffer (20 mM sodium citrate pH 5.0, 1 mM EDTA, 8 M urea, and 0.025% XC and BPB) at 50 °C for 5 min then loading onto the same gel. The gel was prerun at 200 V for 1 hour and run at 200 V for 14 hours. The gel was soaked in 500 ml solution of 5% acetic acid, 5% glycerol and 15% methanol for 30 min to remove urea, then dried at 70 °C for 70 min. The RNase H cleavage was visualized after 2 days of exposure on Kodak film.

Results and Discussion:

Peptide Synthesis on PEG.

A general procedure for liquid phase peptide libarary synthesis was established. The new feature is the disulfide bond link between peptides and the PEG support which is stable during TFA treatment. The PEG linked peptide can be used directly in screening by gel-shift assay in which enough gel retardation can be obtained. The disulfide bond can be cleaved by reducing reagent, i.e. DTT. The peptide can be identified and later connected to an oligonucleotide.

PEG-OPSS reacted readily with the thiol group on cysteamine in aqueous solution at pH7. The excess cysteamine and the leaving thiopyridine group were separated from PEG by SEC column (Fig. 1). During the peptide synthesis, each coupling/deprotection product was purified by ethyl ether precipitation, but the precipatate alone after each coupling was not sufficient to remove the excess reagents, Fmoc amino acid and HOBt, which co-precipatated with addition of ether.

Therefore, an LH-20 column was used to separate most of the unreacted reagents from PEG-peptide prior to the ether precipatation. For peptide library synthesis, only the coupling reactions needed to be done separately, all the other steps could be done as one mixture. Since the reaction is in liquid phase, each step of mixing and separation was done homogeneously.

Preparation of mRNA.

Restriction enzyme digestion of the plasmid showed the presence of the correct gene (Fig. 2). Sal I cleavage gave the linearized plasmid. Xba I cleavage gave the 4.1 kb of c-erb-b2 gene insert and 7.1kb pCHC6 vector. Primers were chosen for PCR in order to generate 5-end mRNA right before the AUG.

The primer sequences for generating PCR products with T7 promoter sequence are:

```
5' primer:
          T7 promoter
TAA TAC GAC TCA CTA TAG CAC CAT GGA GCT GGC GGC CTT G
                     169     AUG

3' primers:
GTC CTC AAA GAG CTG GGT GC                              337-mer
505                       485

              G CTG GGT GCC TCG CAC AAT CC              328-mer
              494                         473
```

The melting temperatures (Tm) for the primers are 63 - 68 ^{o}C. PCR was optimized by different thermal cycles and magnesium concentrations. Analytical agarose gel show the right PCR products (about 350nt) (Fig. 3). A fragment of HER-2/neu mRNA was made by *in vitro* transcription. Denatured PAGE showed its correct size (Fig. 4). In order to get the efficient labeling, RNA had to be purified and dephosphorylated before it could be end labled by radioactive γ - [^{32}P]-ATP using T4

Kinase (Fig. 5). Nuclease T1 and PhyM cleavage bands showed the correct sequnence (Fig. 6 lane 1 &2).

Mapping the RNA sensitive sites for cleavage using oligonucleotide libraries by RNase H assay.

Four 5-mer oligonucleotide libraries, were synthesized through DNA Synthesis Lab.

Lib-A: XXAXX X means mixing site of four nucleotides

Lib-C: XXCXX

Lib-G: XXGXX

Lib-T: XXTXX

Fig. 6 shows the cleavage pattern with these different libraries. Several sensitive regions were found. Especially sensitive to the RNase stimulating activity of a complementary 5-mer DNA was the RNA sequence from 250-256 (Fig. 6). Indeed, complete cleavage of mRNA fragment was obtained using DNA 5-mers complementary to this sensitive site (Fig. 6). It was observed that not only are there limited sites capable of being cleaved, but also complete cleavage of the mRNA fragment can be obtained using a 5-mer DNA at low micromolar concentrations. When nanomolar concentrations of these 5-mers were used, no cleavage was observed (data not shown). Thus, the challenge of this project for the coming year is to find a peptide that can be linked to a 5-mer DNA and allow it to stimulate RNase H cleavage in the nanomolar concentration range.

Conclusions:

Task 1. The new procedure for synthesizing peptides om thiol-derivatized PEG has been developed. It requires using size exclusion chromatography for removing reagents at each cycle of synthesis. This is inconvenient, but does not pose any

technological problem. The ether precipitation procedure is convenient for concentrating the sample at any point during synthesis.

Task 2. Fragments of HER-2/neu mRNA have been prepared and all the necessary techniques have been accomplished. In addition, the preliminary RNase H mapping study supports the feasibility of our hypothesis.

Task 3. Preliminary gel shift experiments hsve not been done due to time lost in an unfortunate personal problem. However, the gel shift procedure had been developed previously by Dr. Wang on a different RNA target.

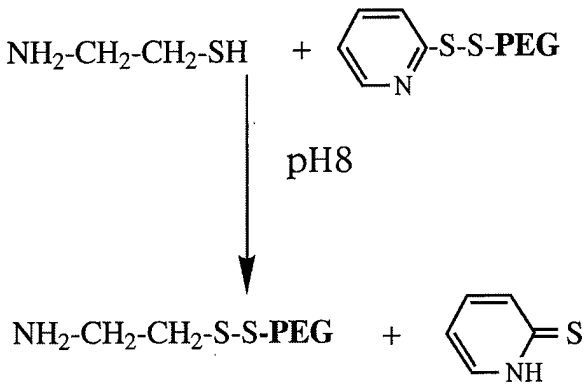
References

1. Tung, C.-H., Wang, J., Leibowitz, M. J. and Stein, S. Dual specificity interactions of HIV-1 TAR RNA with Tat peptide-oligonucleotide conjugates. Bioconj. Chem. 6, 292-295 (1995).

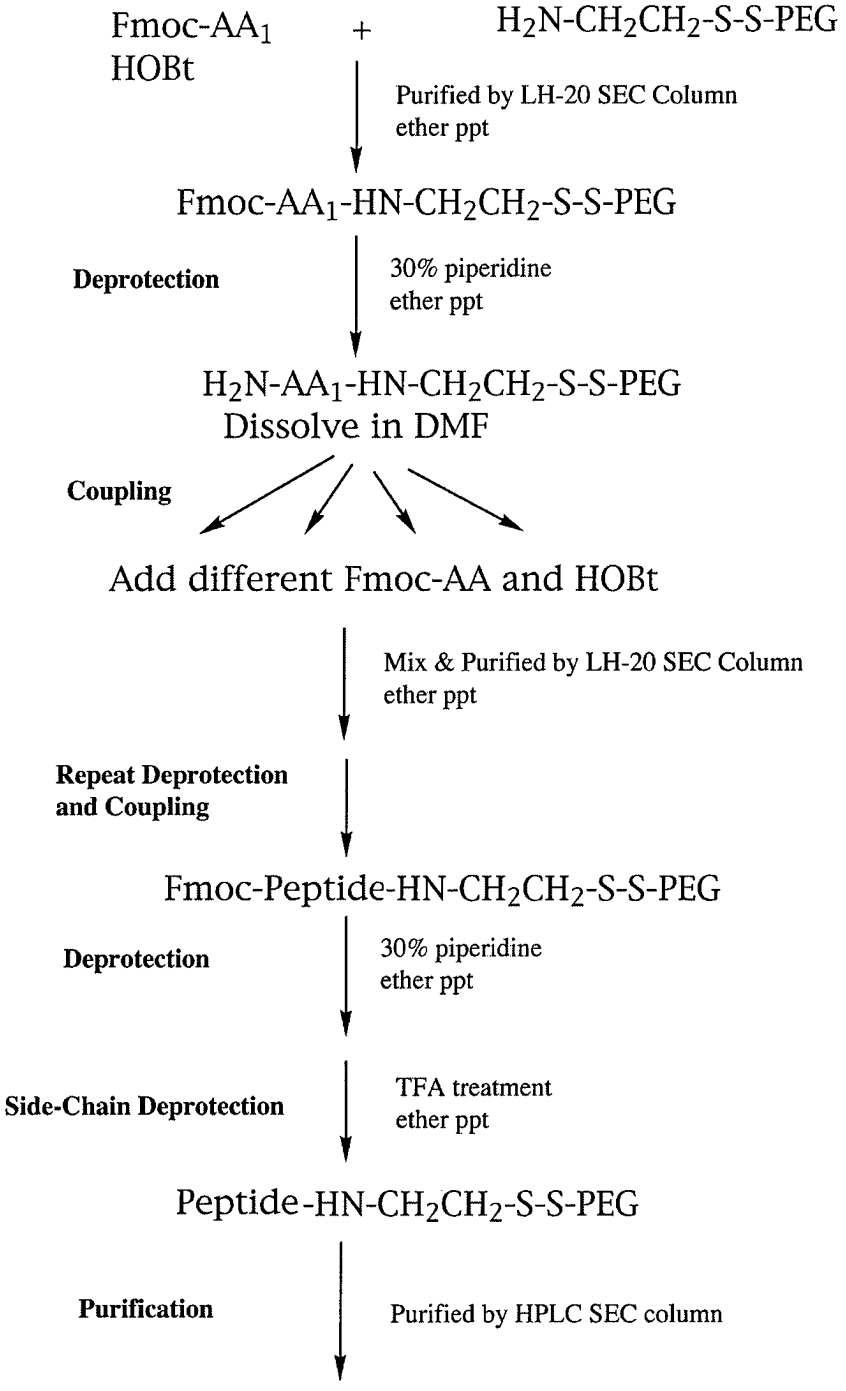
2. Wang, J., Huang, S.-Y., Choudhury, I., Leibowitz, M. J. and Stein, S. Use of a polyethylene glycol peptide conjugate in a competion gel shift assay for screening potential antagonists of HIV-1 Tat protein binding to TAR RNA. Anal. Biochem. 232, 238-242 (1995).

3. Liu et al. Breast Cancer Resaerch and Treatment 34: 97-177, 1995.

Scheme 1. Attachment of Cysteamine to PEG-OPSS



Scheme 2. Routes for Liquid-Phase Peptide Library Synthesis



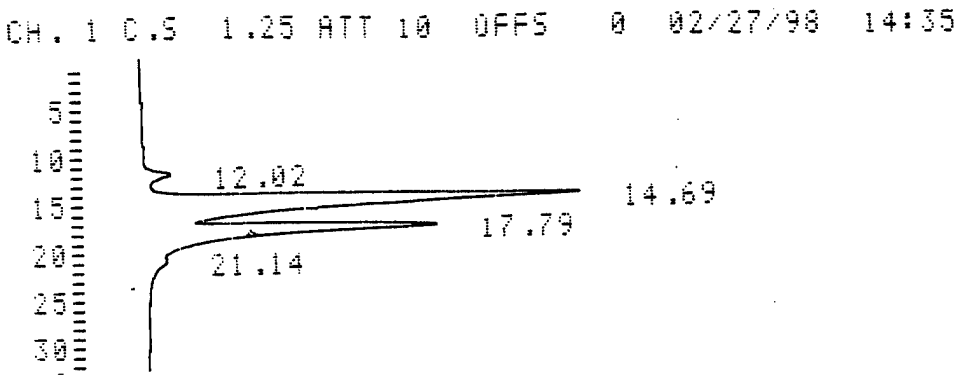


Fig. 1. HPLC profile of purification of PEG-derivative by SEC column.

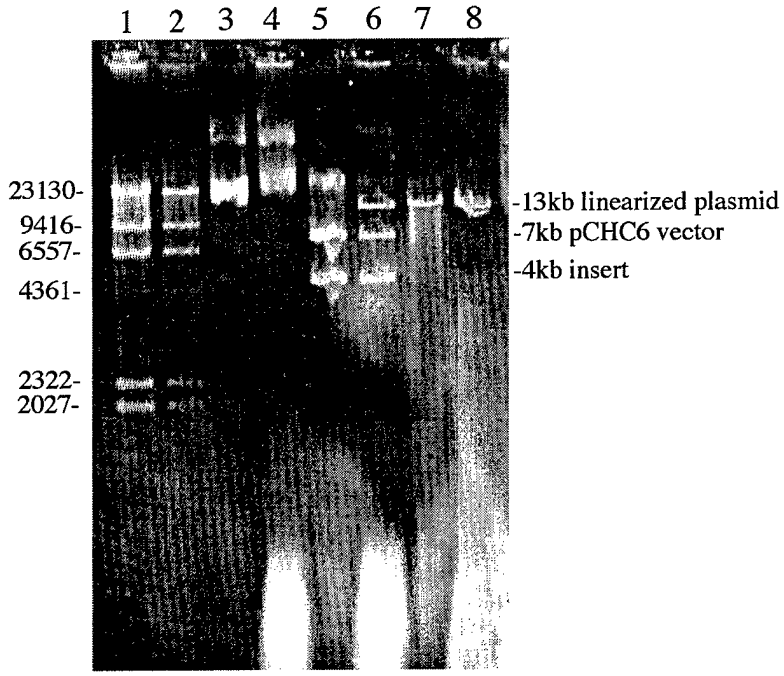


Fig. 2. Restriction Enzyme Digestion of plasmid pCHC6-c-erb-b2.
Lane 1 & 2: lambda DNA/ HindIII fragments.
Lane 3 & 4: whole Plasmid.
Lane 5 & 6: XbaI digestion. Lane 7 & 8: Sal I digestion.
Lane 3, 5, & 7: Large prep of plasmids.
Lane 4, 6, & 8: Small prep of plasmids.

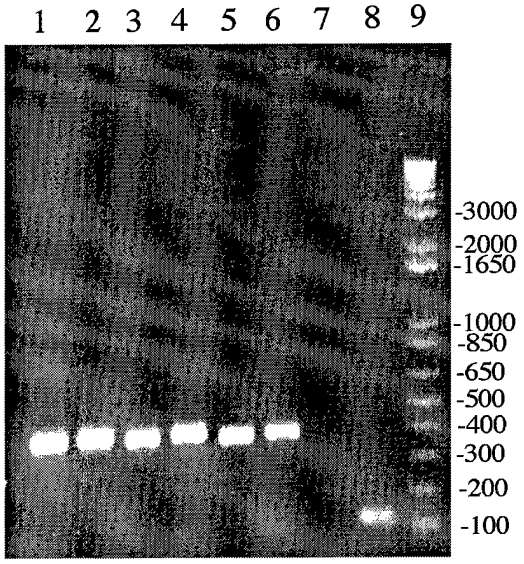


Fig. 3. Analytical Agarose gel of PCR products.

Lane 1-6: PCR products with different Magnesium concentration.
Lane 1, 3, 5: primer 169 and primer 494.
Lane 2, 4, 6: primer 169 and primer 505.
Lane 7: Negative control. Lane 8: Positive control.
Lane 9: 1 kb plus DNA ladder.

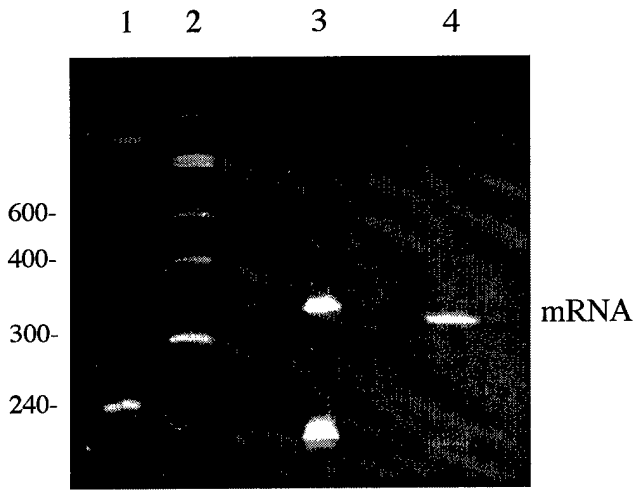


Fig. 4. Analytical PAGE of HER2/neu mRNA transcripts.

Lane 1 & 2: RNA markers.
Lane 3 & 4: RNA generated by in vitro transcription.

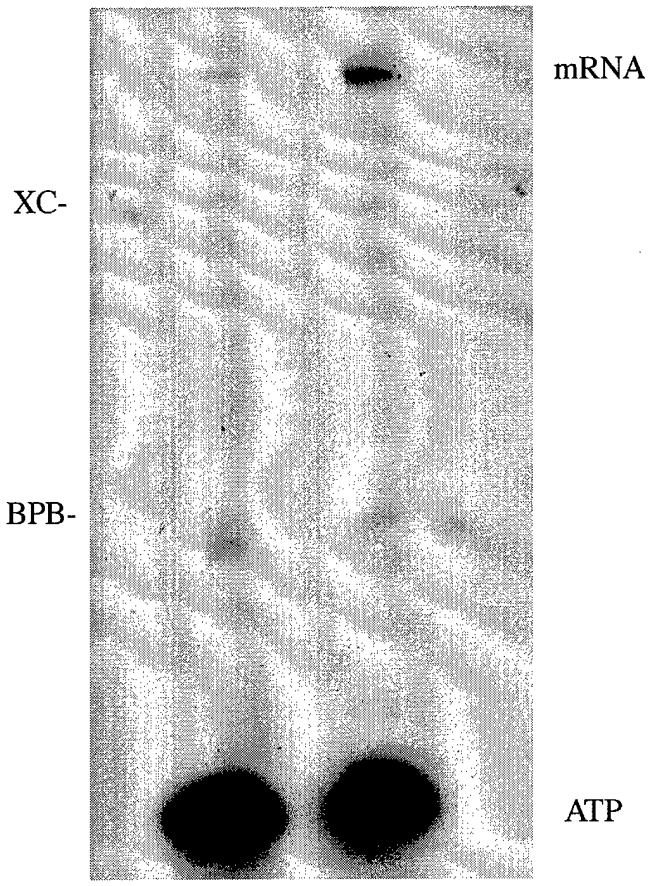


Fig. 5. Kinase Labeling of HER-2/neu mRNA.

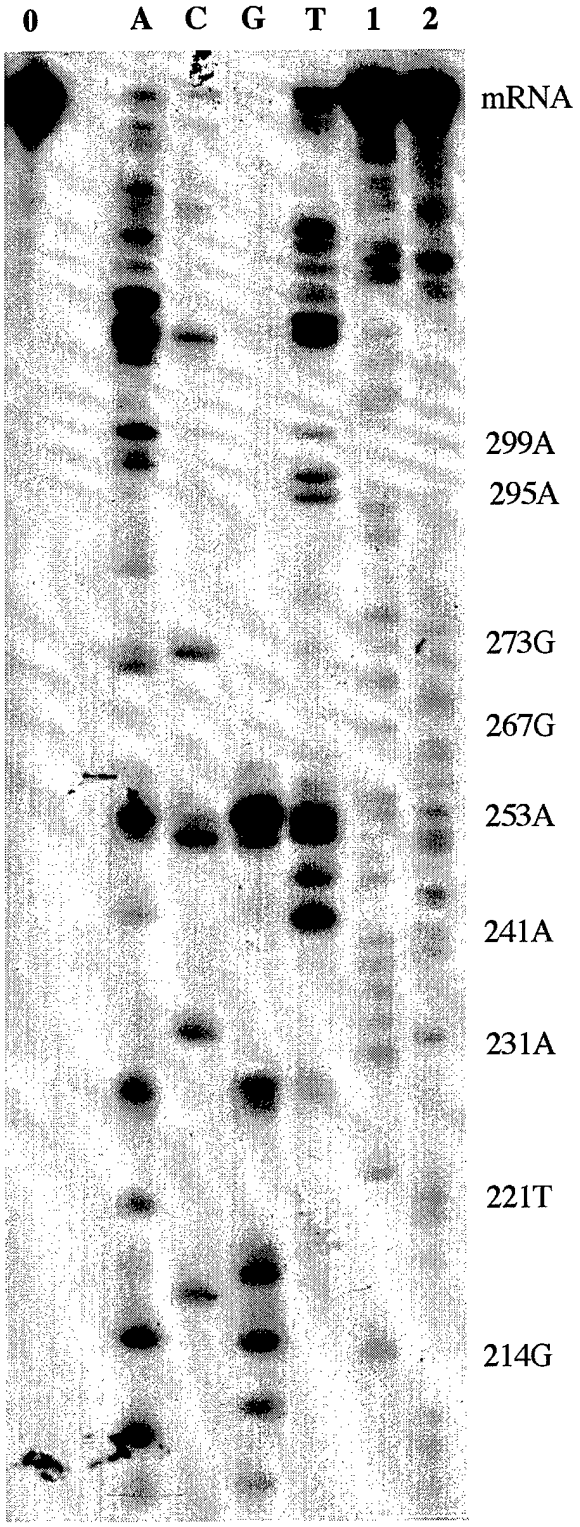


Fig. 6. RNase H Cleavage Assay.

Appendix: Sequence of HER-2/neu gene

```
LOCUS       HSERB2R      4473 bp    RNA      PRI       30-MAR-1995
DEFINITION  Human c-erb-B-2 mRNA.
ACCESSION   X03363
NID         g31197
KEYWORDS    cell surface glycoprotein; cellular oncogene; erB-2
            cellular; glycoprotein; growth factor receptor;
            kinase; neu cellular oncogene; transmembrane protein;
            tyrosine kinase.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa;
            Chordata; Vertebrata; Eutheria; Primates; Catarrhini;
            Hominidae; Homo.
REFERENCE   1  (bases 1 to 4473)
  AUTHORS   Yamamoto,T., Ikawa,S., Akiyama,T., Semba,K.,
            Nomura,N., Miyajima,N., Saito,T. and Toyoshima,K.
  TITLE     Similarity of protein encoded by the human c-erb-B-2
            gene to epidermal growth factor receptor
  JOURNAL   Nature 319 (6050), 230-234 (1986)
  MEDLINE   86118663
REFERENCE   2  (bases 1 to 4473)
  AUTHORS   Papewalis,J., Nikitin,A.Yu. and Rajewsky,M.F.
  TITLE     G to A polymorphism at amino acid codon 655 of the
            human erbB-2/HER2 gene
  JOURNAL   Nucleic Acids Res. 19 (19), 5452 (1991)
  MEDLINE   92020265
COMMENT     The c-erb-B-2 protein shows similarity to the
            epidermal growth factor receptor.
BASE COUNT      902 a   1383 c   1329 g    859 t
ORIGIN
        1  AAGGGGAGGT AACCCTGGCC CCTTTGGTCG GGGCCCCGGG CAGCCGCGCG
       51  CCCCTTCCCA CGGGGCCCTT TACTGCGCCG CGCGCCCGGC CCCCACCCCT
      101  CGCAGCACCC CGCGCCCCGC GCCCTCCCAG CCGGGTCCAG CCGGAGCCAT
      151  GGGGCCGGAG CCGCAGTGAG CACCATGGAG CTGGCGGCCT TGTGCCGCTG
      201  GGGGCTCCTC CTCGCCCTCT TGCCCCCCGG AGCCGCGAGC ACCCAAGTGT
      251  GCACCGGCAC AGACATGAAG CTGCGGCTCC CTGCCAGTCC CGAGACCCAC
      301  CTGGACATGC TCCGCCACCT CTACCAGGGC TGCCAGGTGG TGCAGGGAAA
      351  CCTGGAACTC ACCTACCTGC CCACCAATGC CAGCCTGTCC TTCCTGCAGG
      401  ATATCCAGGA GGTGCAGGGC TACGTGCTCA TCGCTCACAA CCAAGTGAGG
      451  CAGGTCCCAC TGCAGAGGCT GCGGATTGTG CGAGGCACCC AGCTCTTTGA
      501  GGACAACTAT GCCCTGGCCG TGCTAGACAA TGGAGACCCG CTGAACAATA
      551  CCACCCCTGT CACAGGGGCC TCCCCAGGAG GCCTGCGGGA GCTGCAGCTT
      601  CGAAGCCTCA CAGAGATCTT GAAAGGAGGG GTCTTGATCC AGCGGAACCC
      651  CCAGCTCTGC TACCAGGACA CGATTTTGTG GAAGGACATC TTCCACAAGA
```

```
 701  ACAACCAGCT GGCTCTCACA CTGATAGACA CCAACCGCTC TCGGGCCTGC
 751  CACCCCTGTT CTCCGATGTG TAAGGGCTCC CGCTGCTGGG GAGAGAGTTC
 801  TGAGGATTGT CAGAGCCTGA CGCGCACTGT CTGTGCCGGT GGCTGTGCCC
 851  GCTGCAAGGG GCCACTGCCC ACTGACTGCT GCCATGAGCA GTGTGCTGCC
 901  GGCTGCACGG GCCCCAAGCA CTCTGACTGC CTGGCCTGCC TCCACTTCAA
 951  CCACAGTGGC ATCTGTGAGC TGCACTGCCC AGCCCTGGTC ACCTACAACA
1001  CAGACACGTT TGAGTCCATG CCCAATCCCG AGGGCCGGTA TACATTCGGC
1051  GCCAGCTGTG TGACTGCCTG TCCCTACAAC TACCTTTCTA CGGACGTGGG
1101  ATCCTGCACC CTCGTCTGCC CCCTGCACAA CCAAGAGGTG ACAGCAGAGG
1151  ATGGAACACA GCGGTGTGAG AAGTGCAGCA AGCCCTGTGC CCGAGTGTGC
1201  TATGGTCTGG GCATGGAGCA CTTGCGAGAG GTGAGGGCAG TTACCAGTGC
1251  CAATATCCAG GAGTTTGCTG GCTGCAAGAA GATCTTTGGG AGCCTGGCAT
1301  TTCTGCCGGA GAGCTTTGAT GGGGACCCAG CCTCCAACAC TGCCCCGCTC
1351  CAGCCAGAGC AGCTCCAAGT GTTTGAGACT CTGGAAGAGA TCACAGGTTA
1401  CCTATACATC TCAGCATGGC CGGACAGCCT GCCTGACCTC AGCGTCTTCC
1451  AGAACCTGCA AGTAATCCGG GGACGAATTC TGCACAATGG CGCCTACTCG
1501  CTGACCCTGC AAGGGCTGGG CATCAGCTGG CTGGGGCTGC GCTCACTGAG
1551  GGAACTGGGC AGTGGACTGG CCCTCATCCA CCATAACACC CACCTCTGCT
1601  TCGTGCACAC GGTGCCCTGG GACCAGCTCT TTCGGAACCC GCACCAAGCT
1651  CTGCTCCACA CTGCCAACCG GCCAGAGGAC GAGTGTGTGG GCGAGGGCCT
1701  GGCCTGCCAC CAGCTGTGCG CCCGAGGGCA CTGCTGGGGT CCAGGGCCCA
1751  CCCAGTGTGT CAACTGCAGC CAGTTCCTTC GGGGCCAGGA GTGCGTGGAG
1801  GAATGCCGAG TACTGCAGGG GCTCCCCAGG GAGTATGTGA ATGCCAGGCA
1851  CTGTTTGCCG TGCCACCCTG AGTGTCAGCC CCAGAATGGC TCAGTGACCT
1901  GTTTTGGACC GGAGGCTGAC CAGTGTGTGG CCTGTGCCCA CTATAAGGAC
1951  CCTCCCTTCT GCGTGGCCCG CTGCCCCAGC GGTGTGAAAC CTGACCTCTC
2001  CTACATGCCC ATCTGGAAGT TTCCAGATGA GGAGGGCGCA TGCCAGCCTT
2051  GCCCCATCAA CTGCACCCAC TCCTGTGTGG ACCTGGATGA CAAGGGCTGC
2101  CCCGCCGAGC AGAGAGCCAG CCCTCTGACG TCCATCATCT CTGCGGTGGT
2151  TGGCATTCTG CTGGTCGTGG TCTTGGGGGT GGTCTTTGGG ATCCTCATCA
2201  AGCGACGGCA GCAGAAGATC CGGAAGTACA CGATGCGGAG ACTGCTGCAG
2251  GAAACGGAGC TGGTGGAGCC GCTGACACCT AGCGGAGCGA TGCCCAACCA
2301  GGCGCAGATG CGGATCCTGA AAGAGACGGA GCTGAGGAAG GTGAAGGTGC
2351  TTGGATCTGG CGCTTTTGGC ACAGTCTACA AGGGCATCTG GATCCCTGAT
2401  GGGGAGAATG TGAAAATTCC AGTGGCCATC AAAGTGTTGA GGGAAAACAC
2451  ATCCCCCAAA GCCAACAAAG AAATCTTAGA CGAAGCATAC GTGATGGCTG
2501  GTGTGGGCTC CCCATATGTC TCCCGCCTTC TGGGCATCTG CCTGACATCC
2551  ACGGTGCAGC TGGTGACACA GCTTATGCCC TATGGCTGCC TCTTAGACCA
2601  TGTCCGGGAA AACCGCGGAC GCCTGGGCTC CCAGGACCTG CTGAACTGGT
2651  GTATGCAGAT TGCCAAGGGG ATGAGCTACC TGGAGGATGT GCGGCTCGTA
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2751  CAAAATTACA GACTTCGGGC TGGCTCGGCT GCTGGACATT GACGAGACAG
2801  AGTACCATGC AGATGGGGGC AAGGTGCCCA TCAAGTGGAT GGCGCTGGAG
2851  TCCATTCTCC GCCGGCGGTT CACCCACCAG AGTGATGTGT GGAGTTATGG
2901  TGTGACTGTG TGGGAGCTGA TGACTTTTGG GGCCAAACCT TACGATGGGA
2951  TCCCAGCCCG GGAGATCCCT GACCTGCTGG AAAAGGGGGA GCGGCTGCCC
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3001  CAGCCCCCCA TCTGCACCAT TGATGTCTAC ATGATCATGG TCAAATGTTG
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3101  TCTCCCGCAT GGCCAGGGAC CCCCAGCGCT TTGTGGTCAT CCAGAATGAG
3151  GACTTGGGCC CAGCCAGTCC CTTGGACAGC ACCTTCTACC GCTCACTGCT
3201  GGAGGACGAT GACATGGGGG ACCTGGTGGA TGCTGAGGAG TATCTGGTAC
3251  CCCAGCAGGG CTTCTTCTGT CCAGACCCTG CCCCGGGCGC TGGGGGCATG
3301  GTCCACCACA GGCACCGCAG CTCATCTACC AGGAGTGGCG GTGGGGACCT
3351  GACACTAGGG CTGGAGCCCT CTGAAGAGGA GGCCCCCAGG TCTCCACTGG
3401  CACCCTCCGA AGGGGCTGGC TCCGATGTAT TTGATGGTGA CCTGGGAATG
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3601  CCAGATGTTC GGCCCCAGCC CCCTTCGCCC CGAGAGGGCC CTCTGCCTGC
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3701  GGAAGAATGG GGTCGTCAAA GACGTTTTTG CCTTTGGGGG TGCCGTGGAG
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3801  TCCTCCTGCC TTCAGCCCAG CCTTCGACAA CCTCTATTAC TGGGACCAGG
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3901  GCAGAGAACC CAGAGTACCT GGGTCTGGAC GTGCCAGTGT GAACCAGAAG
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4001  ACTTCTGCTG GCATCAAGAG GTGGGAGGGC CCTCCGACCA CTTCCAGGGG
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4101  TTCCCAGATG GCTGGAAGGG GTCCAGCCTC GTTGGAAGAG GAACAGCACT
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4201  AGTGGATGCC ACAGCCCAGC TTGGCCCTTT CCTTCCAGAT CCTGGGTACT
4251  GAAAGCCTTA GGGAAGCTGG CCTGAGAGGG GAAGCGGCCC TAAGGGAGTG
4301  TCTAAGAACA AAAGCGACCC ATTCAGAGAC TGTCCCTGAA ACCTAGTACT
4351  GCCCCCCATG AGGAAGGAAC AGCAATGGTG TCAGTATCCA GGCTTTGTAC
4401  AGAGTGCTTT TCTGTTTAGT TTTTACTTTT TTTGTTTTGT TTTTTTAAAG
4451  ATGAAATAAA GACCCAGGGG GAG
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